Cultured Neuron Probe

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General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Summary

During the period of this report a variety of *in vitro* studies were made. SCG experiments were done to study the phototoxicity of DiI staining, which was found to be similar to that for hippocampal cells; electrical recording experiments were done; experiments were made to study the escape of hippocampal neurons from wells with redesigned grillwork; and techniques were evolved for loading DiI stained hippocampal cells into dummy probes.

Using the first complete neurochips, the problem of electrophysiological recording was attacked. It was decided to study neurochip electrode signals while intracellularly recording from and stimulating cells in wells, as the most direct verification of well function. To do this, bent electrodes were fabricated, and shown to be as good as straight ones in control cultures. An electrode testing procedure was developed to verify the electronic integrity of the system before trying to impale cells, and software was modified to provide effective elimination of 60 herz pickup, and signal averaging if desired.

First attempts at recording from well electrodes were not successful, mainly because cell penetrations did not work well. The population of cells for testing was small, because long term survival was poor, as well as because of escape from the wells. A single case of a successful stimulation of a cell in a well was rendered ineffective by a shunt capacitance problem which is still not understood. During the coming quarter it is expected that more cells will be available for these tests and that they will be successful.

The new grillwork design described in the last quarterly report was tried with a variety of hole sizes, and unfortunately escape was seen even through the smallest aperture, which was 1 x 3 μm. As a result of these observations, a new grillwork design was invented, in which the strategy for cell containment was changed from a small aperture at the edge of the well to long tunnels from which processes must emerge from the well. A mask set was made and fabrication begun on a new set of dummy chips (without electrodes) to test for outgrowth and escape with the new design. Tunnels of these test chips will have lengths of 15 and 30 microns, and cross sections from .2 x 1.5 to 3 x 4 microns. Tests with these chips will be made during the next quarter.

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Dummy probes were made for *in vivo* experiments, incorporating the zero-overhang well design which allows good outgrowth. The process of learning how best to load them with DiI stained neurons was pursued at Caltech, and a successful methodology was developed for use at Rutgers. Experiments with these probes will be done next quarter. It is hoped that short-term experiments will yield information about initial neuron outgrowth and survival in the rat, as well as whether escape from the wells *in vivo* has the same likelihood as *in vitro*.

Neurochips

SCG experiments

DiI staining and phototoxicity

We have previously reported protocols for DiI staining of SCG and hippocampal neurons in suspension in order to enhance visualization of fine processes. In the last report, we showed that hippocampal neurons stained with DiI were killed by exposures to the mercury arc lamp as short as 10 seconds. We have now seen that SCG neurons are similarly susceptible to phototoxicity. SCG neurons were stained with DiI before plating onto a culture dish with a labeled grid allowing unambiguous location of individual neurons. Particular neurons on one- and three-day-old stained cultures were exposed to the arc lamp for 10 to 60 seconds. After a day, the formerly healthy neurons had either disappeared or looked decidedly unhealthy (lots of vacuoles in somata, shriveled edges). Other neurons in the same dish that had not been exposed to the arc lamp looked perfectly healthy.

Electrophysiology on cells in wells

In order to test the neurochips, we need to be able to record intracellularly from neurons in wells while simultaneously recording from the neurochip electrode. Getting a standard intracellular electrode into a well can be difficult because the objective of the upright microscope limits the steepness of the angle at which the electrode can approach the neuron; but it is easier to get a good recording when the electrode tip is at a steep angle to the substrate. In order to allow a better approach to the neurons, we have started using electrodes that are bent between shoulder and tip, as shown below:

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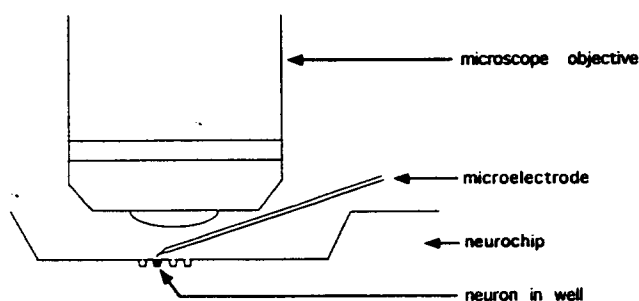
Electrodes are made by pulling glass (1 mm O.D., 0.5 mm I.D., with filament (WPI 1B100F-4)) in the usual way on a P-77 Brown-Flaming horizontal puller (Sutter Instrument Company) to get fine tips. The pipette is positioned at a 45 degree angle over a platinum filament, through which current is passed until it is red-hot. The pipette is then lowered towards the electrode until the tip starts bending, and the current is turned off when the bend is about 60 degrees. The pipette can then be filled with electrolyte solution (3M KCl) by capillary action followed by backfilling to give an electrode with a resistance of about 100 MΩ.

In control cultures on flat dishes, there is no difference in success rate in recording intracellularly using bent or straight electrodes. We have not as yet tried recording from enough neurons in neurochip wells to know whether the bent electrodes make recording in this situation easier.

Neurochip cultures and recording attempts

Since the last progress report, we have grown eight cultures on neurochips whose bond pads had been removed to reduce the shunt capacitance. However, we have not yet successfully recorded action potentials with neurochip electrodes.

Outgrowth and survival of SCG neurochip cultures is extremely variable. Of the eight neurochip cultures with all sixteen wells stuffed with neurons, an average of 7.5 wells (range: 3 to 14) showed outgrowth on the first day. After more than a few days in culture, it becomes difficult to tell whether a particular well contains a growing neuron, because of the proliferation of axons throughout the basin. Also, cell bodies are difficult to see inside wells because their edges disappear as the cell fills the well. After about two weeks in culture, it is again possible to identify cells in wells by the outgrowth of thick dendrites. In the last eight cultures, an average of only three cells per neurochip have remained in wells long enough to grow dendrites. Presumably, the other neurons that grew initially either escaped from their wells or died.

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In all the intracellular electrophysiology we have done on cultured SCG neurons, we have found that the likelihood of recording large action potentials increases with age in culture. It is only after about 10 days in culture that a majority of impaled neurons will produce large spikes. We have therefore been reluctant to try recording from neurochip cultures that are less than two weeks old, because an unsuccessful attempt at intracellular penetration will generally kill the neuron. However, the low long-term survival rate of neurons in wells has not allowed us to make many attempts at recording. Of the eight neurochips loaded with neurons, six either did not survive, or did not have any neurons remaining in wells with active electrodes, by two weeks in culture.

We did attempt to record from the two remaining neurochip cultures. In one case, using bent electrodes on a 13 day old neurochip culture, we were able to record spikes from a neuron inside a well whose electrode was not working. However, we were unable to get successful penetrations into any of the other six presumed cells in wells. It was very difficult to be sure whether there actually were cells in these wells, because they had not yet grown dendrites.

In our second recording attempt, on a 15 day old culture, we managed to get a stable penetration into one out of four neurons in wells and evoke action potentials. We did not see any signal at the neurochip electrode. Following the recording attempt, the impedance of the electrodes on the chip was measured, and another shunt capacitance was discovered to have attenuated the signal. We are still looking for the source of this capacitance.

A possible explanation for the low survival rate of neurons in wells is the sparseness of the neurochip cultures. SCG neurons do not survive well when cultured in small numbers unless they are cocultured with a larger population of neurons in the same dish which serve to condition the medium. In our original neurochip culture experiments, we supplemented the small population of neurons in the basin with a coverslip carrying a dense "feeder" culture. Later, we simply plated a large number of neurons onto the chip and relied on a sufficient population of neurons on the top surface of the silicon wafer to sustain the smaller number in the basin. However, the latest neurochips have a layer of Sylgard over the top surface of the wafer; SCG neurons do not grow well on

Sylgard. Therefore, we suspect that the poor survival of neurochip cultures may be due to insufficient numbers of neurons to condition the medium. We therefore plan to return to including a coverslip culture in our neurochip dishes.

Electronics Checks for Neurochip Electrodes

In light of our inability to record activity from SCG neurons in wells, the following question has arisen: If a cell fires an action potential in a well, are the electrodes sufficiently sensitive to detect it? Theoretically, our models indicate that such sensitivity is possible, but in the absence of experimental confirmation, theory is not helpful.

First, we measured the actual sensitivity of the entire amplifier system by putting a known signal from a function generator into the pre-amplifier input, and measured the A/D converter output. We found a conversion factor of 1.8 μV per A/D count, similar to the value expected given the design of the circuits. We greatly reduced the noise level in the system by eliminating a ground loop and several external 60 Hz generators, by installing a crude Faraday cage around the microscope, and by shielding the cables running between the pre-amplifier and the amplifiers. This resulted in zero 60 Hz noise and 5 counts RMS (9 μV RMS) in a 400 kΩ dummy load, and 20 counts RMS (38 μV RMS) 60 Hz noise and 5 counts RMS (9 μV RMS) in an open circuit.

Next, we modified the software to average away the remaining 60 Hz noise. Since the data recording is time-locked to 60 Hz, the phase of the 60 Hz noise is fixed and can be subtracted away. The program now keeps a running average of the background signal, incremented on demand by an adjustable fraction of the current signal. This is essentially an exponentially-weighted running average. Once a background level is established, the averaging can be suspended while displaying the difference between the average background and the current signal. When used properly, this procedure can theoretically eliminate any amount of 60 Hz noise, and can also eliminate stimulus artifacts time-locked to the A/D converter. In practice, however, the 60 Hz noise tends to drift somewhat, so it is best to reduce the noise as much as possible, using the background averager to clean up the small amount remaining.

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We then incorporated a signal averager after the background averager. Using the same exponential averager principle, repetitive signals time-locked to the A/D converter can be averaged to increase the signal/noise ratio. Theoretically, all random noise can be averaged to zero by sampling long enough, since the noise decreases as $N^{-1/2}$ (where N is the number of traces averaged). In addition, if the noise can be averaged to less than one A/D count, the effective number of bits in the A/D converter can be increased.

Using these new features, we used a submicron pipette to inject current into a neurochip well filled with saline. We estimate that an SCG cell body should sink 5-10 nA of current during an action potential, so we injected a 5 nA square pulse. The following figure illustrates the output of the LabView program digitizing the data. The length of the x-axis is 100 msec, and the units are 0.2 msec long. The y-axis scale is 50 counts. A -5 nA, 45 msec long square pulse was injected into one well. The response of the electrode is biphasic, as expected from a capacitive sensor, with a -25 count pulse at 16 msec and a +25 count pulse at 60 msec. The noise level is approximately 3-4 counts RMS. Thus, we can conclude that the well electrodes should easily be able to detect an action potential from an SCG neuron in a well.

13:00:02 4/18/95

Stimulus Length(ms) 20.0
Stimulus Delay(ms) 70.0

0.8 0.8 0.8 0.8
0.8 0.8 0.8 0.8
0.8 0.8 0.8 0.8
0.8 0.8 0.8 0.8

caption

Q6. SCG's 15 days in culture. Poking cell in #3. Can see the -5 nA stimulus at 80 and 300

Run
Stop

1 2 4 8

FileName
Joule 1050-Q.:Users.:Mike:LVData:4/18/9513.

Rate (Hz)

9: 8: 7: 6:
11:

0 0 20 30 40 50   0 0 20 30 40 50   0 0 20I 30 40 50   0 0 20 30 40 50

1.0
0.5
0.0
-0.5
-1.0

0.9
0.4
-0.1
-0.6
-1.1

3:

0 100 200 300 400 500

Record Stimulate
50   30000

FILTER
Time const
0

Record
Delay(ms) 67.0   Rate(kHz) 5.0   Length(ms) 100.0
0-7 Error   8-15 Error

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Hippocampal culture experiments

Outgrowth and escape from dummy wells with new grillwork designs

Dummy chips with four new grillwork designs as described in the previous report were fabricated. We loaded wells with almost all combinations of grillwork design and feature size with dissociated E17 hippocampal neurons in order to determine rates of process outgrowth and cell body escape. The results, summarized in the table below, were mixed, but it is clear that hippocampal neurons can move through even the smallest holes on all of the grillwork designs.

Chip	Grillwork design	Feature size	Number of wells with initial outgrowth (out of 16)	Number of neurons escaping	Lifespan of culture (days)	Comments (see below)
GT7	Diamond 2	3 μm	14	8	16	a
		5 μm	10	1	16	b
		8 μm	14	4	16	
GT3	Diamond 1	3 μm	12	0	8	c
		5 μm	3	0	8	c
		8 μm	10	0	8	c
GT7	Grid	4 μm	4	1	2	d
		5 μm	10	1	2	d
		6 μm	11	1	2	d
GT4	Diamond 1	3 μm	11	3	4	e, f, i
		5 μm	6	1	4	f, i
		8 μm	10	4	4	f, i
GT1	Diamond 2	3 μm	7	1	2	g
		5 μm	6	1	2	g
		8 μm	10	2	2	g
GT5	Circle	3 μm	7	1	2	h, i
		4 μm	6	1	2	h
		10 μm	6	0	2	h

a) One cell escaped on the third day in culture, 6 on the 9th day, and one on the 14th day
b) Although this culture was in the same dish as the above-described 3 μm grillwork, most of the processes in the well area disappeared by day 9
c) This culture as well as its siblings in plastic culture dishes grew very poorly for unknown reasons

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d) Virtually all cells in this dish were dead by the third day in culture, possibly because the culture was very sparse to begin with; sibling control cultures were fine
e) Only 7 wells had processes by day 2
f) The premature death of this culture (4 days) was probably due to too low pH, due to residual air medium left in the dish after observation
g) culture was contaminated with bacteria on the third day in culture
h) culture was inadvertently killed by the addition of a concentrated salt solution to the dish
i) see photos on following pages

As can be seen from the results in the table, outgrowth from wells with all grillwork types was quite good, with an average of 8.7 out of 16 neurons per well array growing. However, for all but one combination of grillwork design and feature size, at least one neuron escaped through the grillwork. In most cases only a single neuron escaped; however, it seems likely that had the cultures lived longer, more neurons would have escaped. On chip GT7, with Diamond 2 grillwork, 10 out of 28 presumed healthy neurons eventually escaped.

The photos on the following pages show some examples of escaping neurons. There appear to be two patterns of escape. In some cases, the cell body appears to ooze through a grillwork opening, first appearing as a small blob of cytoplasm and then forming a larger cell body. In other cases, a thick process is seen to emerge from a well, and it then swells somewhere along its length, apparently re-forming a soma outside the well. It would be interesting to stain such putatively escaped neurons with a nuclear stain to verify that the nucleus did indeed "escape" from the well.
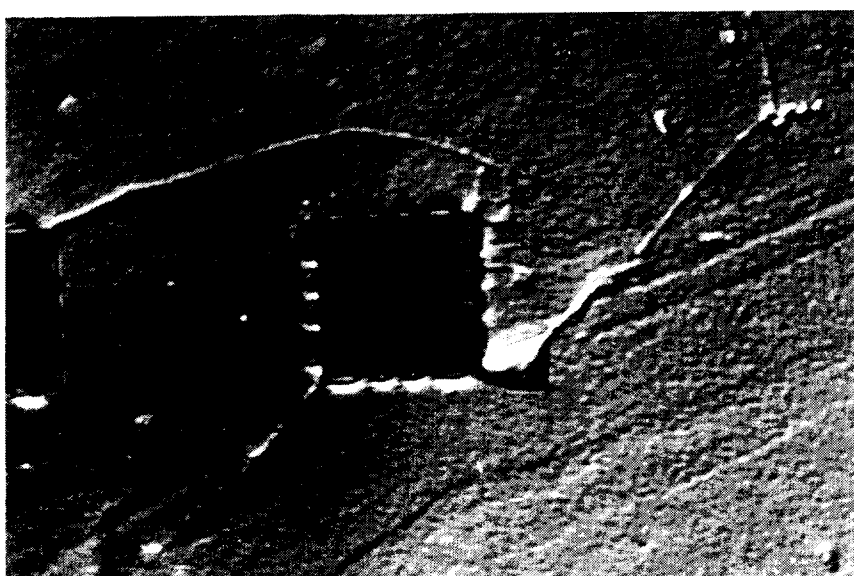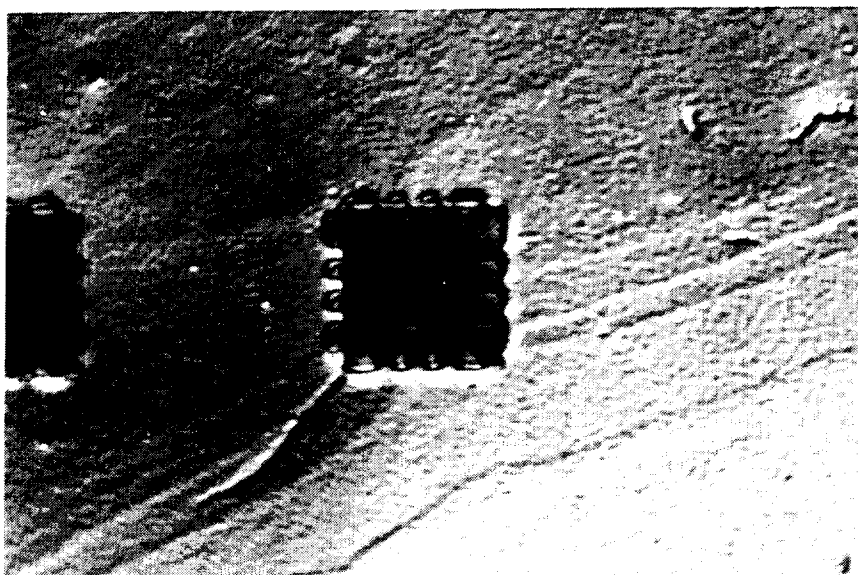
Dummy chip GT5, Circle grillwork, 3 μm feature size. Process outgrowth several hours after plating (top) and escape of the cell body out a corner hole of the same well two days later (bottom).
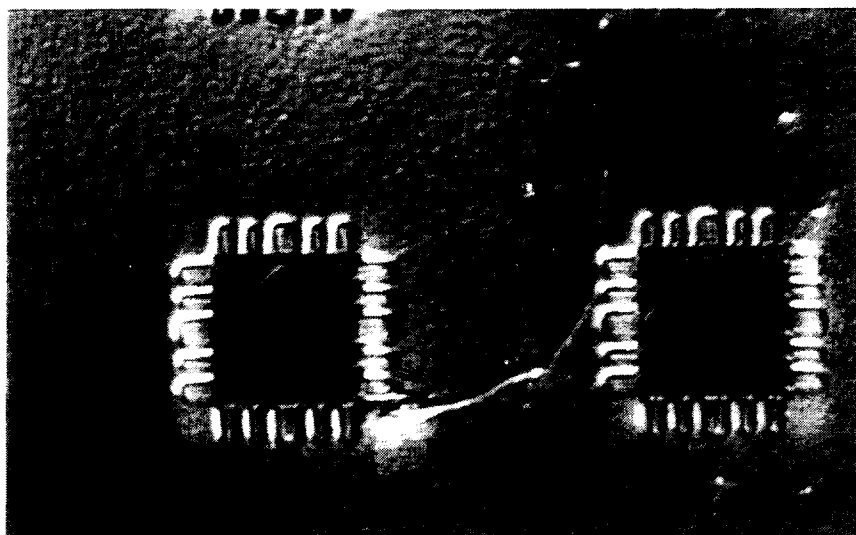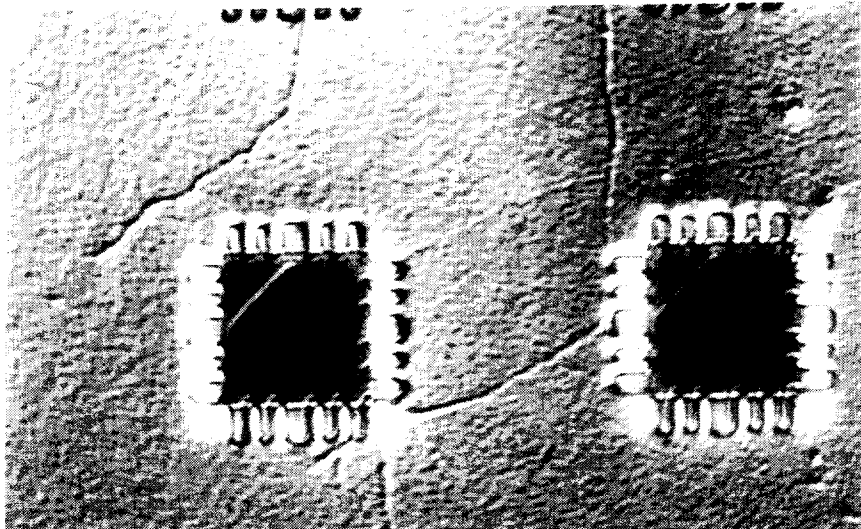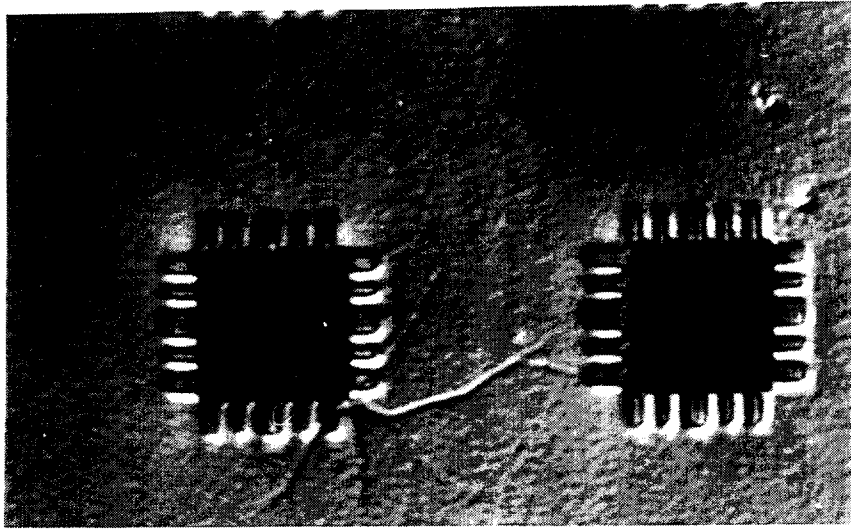
Dummy chip GT4, Diamond 1 grillwork, 3 µm feature size. Outgrowth and escape of a hippocampal neuron . 12 hours (top) , 24 hours (center), and 3 days (bottom) after plating.

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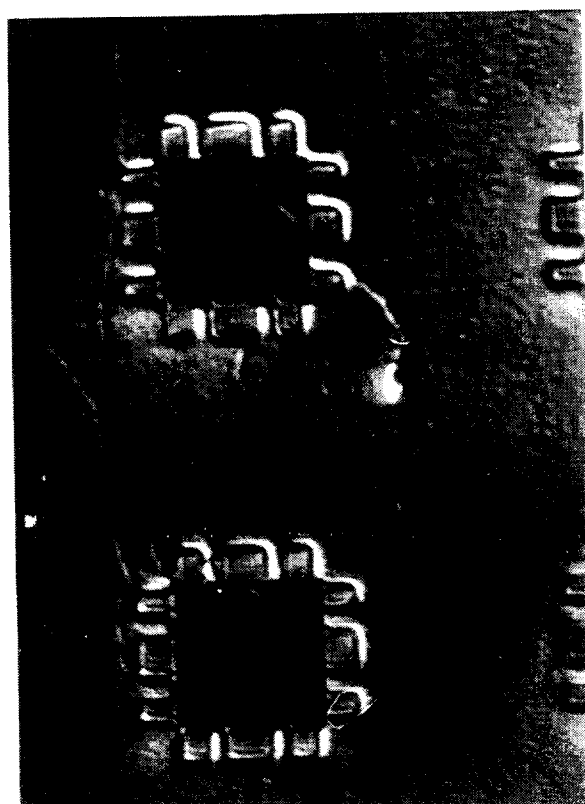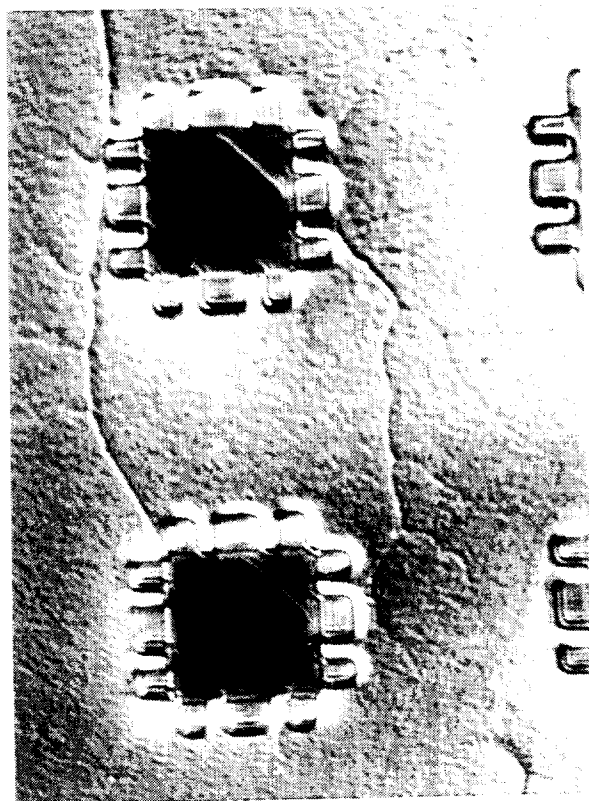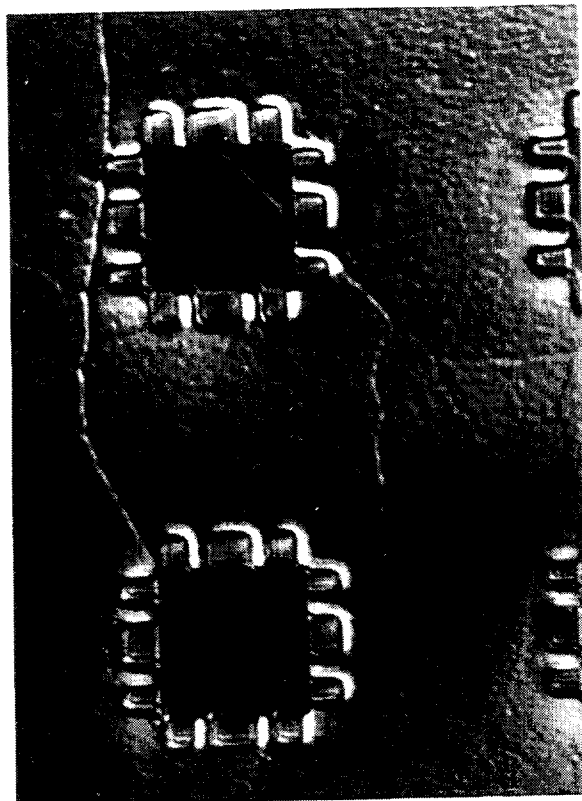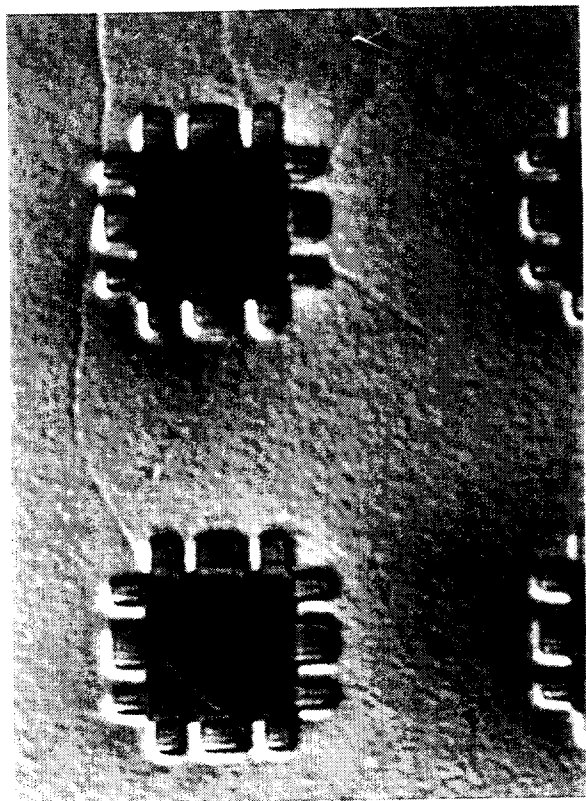
Dummy chip GT4, Diamond 1 grillwork, 5 μm feature size. Outgrowth and escape of a hippocampal neuron. 12 hours (top left), 24 hours (top right), 3 days (bottom left) and 4 days (bottom right) after plating.

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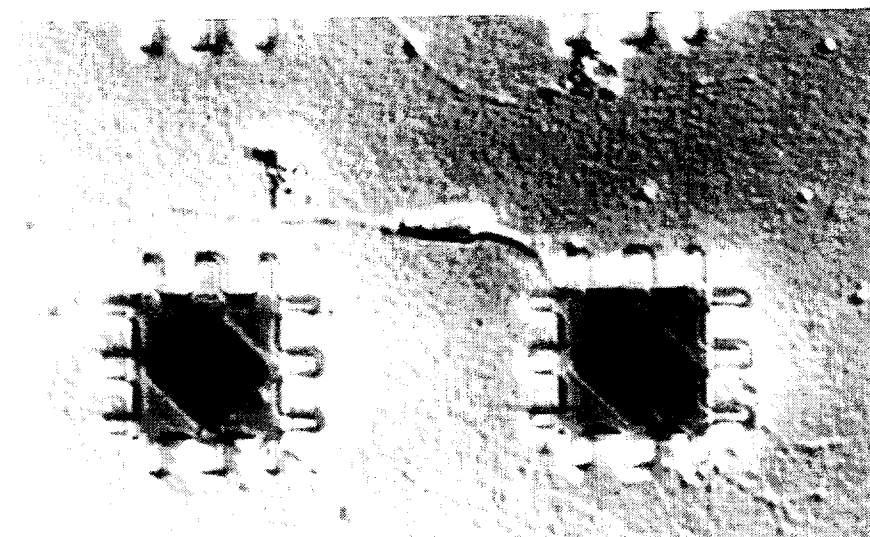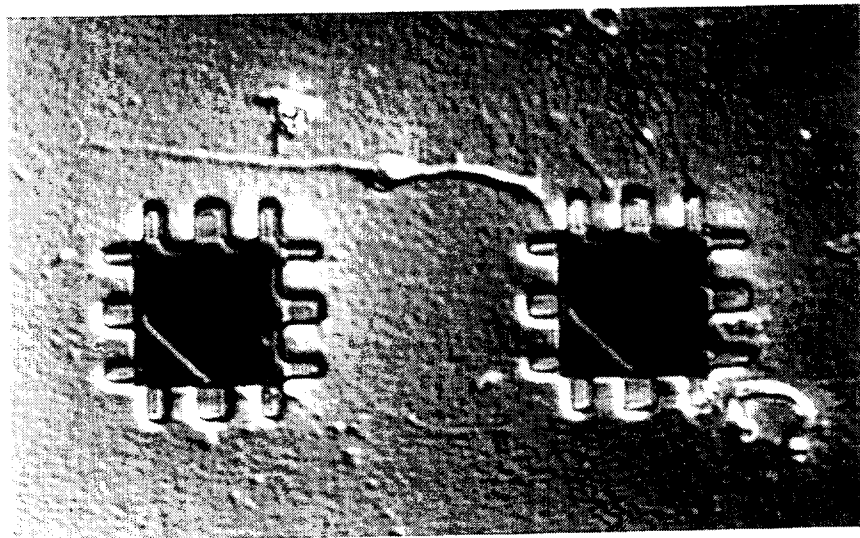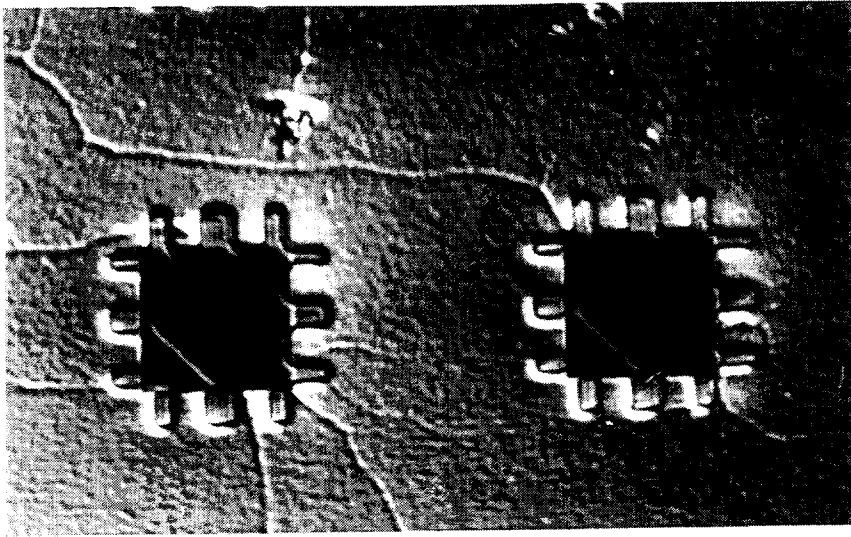
Dummy chip GT4, Diamond 1 grillwork, 8 μm feature size. Outgrowth and escape of a hippocampal neuron. 1 day (top), 3 days (center), and 4 days (bottom) after plating.

Probe Experiments

As a prelude to implanting loaded probes into live rats, we demonstrated that DiI-stained fetal rat hippocampal neurons will live and grow in probe wells. We used the following procedure to stain the neurons in suspension:

(1) Stock staining solution: 40 mg/mL DiI C12 (Molecular Probes C-383) dissolved in 2.5% Pluronic F127 (BASF) in N,N-dimethyl formamide.

(2) Dilute staining solution 1000:1 with medium.

(3) Add cell suspension to staining solution (both at 37 °C).

(4) Incubate 15 minutes at 37 °C.

(5) Add 200 μL 5% bovine serum albumen (BSA) to bottom of tube. Spin 6 minutes at 150g to pellet cells.

(6) Remove all but 50 μL of staining solution and BSA.

(7) Resuspend cells in 1 mL medium.

(8) Repeat steps (5-6) to further rinse cells.

(9) Resuspend cells in 250 μL medium to maximize cell density.

5% BSA is more dense than medium, does not mix readily with medium, and reduces the stickiness of the cells. Using the BSA to separate out the cell pellet allowed us to achieve very high cell recovery rates of 80% or more, and two rinses greatly reduce the background staining.

Probes were prepared with 1 hour soaks in 1 mg/mL poly-DL-lysine in PBS and in 13 μg/mL laminin in PBS. 35,000 unstained neurons were plated onto the dishes and were allowed to grow 1-2 hours as a feeder layer. The dishes were then flooded with 2 mL of medium, and the unstained cells on and near the probes were removed by suction with a 40 μm diameter pipette. Twenty to thirty thousand stained cells were then plated onto the probes. The cell suspension density is typically 10^6 cells/mL, so the volume of stained cell suspension added is of the order of 20 μL, further diluting any DiI left in the cell suspension.

As the cells fell, they were manipulated into position above wells with a pusher pipette and allowed to fall into the well (this method is dubbed

'parachuting'). In this way, the cells were never touched by the pipette, and the first solid surface they encounter is the bottom of the well. Hippocampal neurons are extremely sticky, attaching immediately to the first solid surface they encounter. The attachments formed are extremely robust, and the cells maintain filaments attached to the substrate which can stretch elastically for hundreds of microns. Often, when a cell is touched with a pusher pipette, the cell will stick to it and to the surface, drawing filaments from the cell between attachment points. If the cell touches the grillwork, it will form attachments, and the cell will invariably pull up to the grillwork and escape through the central hole within a few hours. We found that parachuting the cells prevents attachment to the grillwork and increases the probability that the cell will stay in the well.

Cells typically fall for only 10-15 minutes after plating, while loading wells takes 30-45 minutes. Two methods were used to obtain more cells. The first and simplest is to simply plate more stained cells. This requires opening the lid of the dish in a non-sterile environment, introducing the possibility of infection. The second method uses fallen cells, and can be performed while maintaining sterility. A cell which has already fallen to the surface is detached with the cell pusher, shaken free of the pusher above the probe, and then treated as a freshly fallen cell. Care must be taken not to smash the cell, and to remove all attachment filaments before allowing the cell to drift freely. When selecting cells from the population of fallen cells, stained ones were easily detected by brief observation under fluorescence. No difference in cell survival and growth was noted between freshly falling and resuspended cells. To increase the probability of obtaining growth from wells, two cells were loaded into each well in this experiment.

Probe well loading of stained cells was performed under illumination from a tungsten bulb. The stained cells in the probe region are illuminated for periods up to an hour; we noticed some phototoxicity from this irradiation. We therefore now use a red filter (which eliminates all the green excitation light for DiI) in front of the light source whenever stained cells are present.

On four probes that were loaded with DiI stained cells, we obtained growth after two days from 6/16 wells (DP17), 9/16 wells with one escaped cell

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(DP18), 9/16 wells with 4 escaped cells (DP19), and 11/16 wells with 2 escaped cells (DP21). Cell bodies and processes were brightly stained, and background fluorescence was minimal. The figure on the next page is a montage of fluorescence micrographs of DP19 taken after three days in culture. Notice the well-stained processes, the brightly stained cell bodies, the lack of background staining, and the presence of dead, stained cells in wells without processes emerging. Also note that the silicon nitride grillwork is highly transparent.

We have demonstrated that DiI-stained hippocampal neurons can survive in probe wells, and remain brightly stained for at least three days. In the next quarter, we will attempt to lay loaded probes onto slices to observe outgrowth of stained cells into the slice. We will also attempt to implant loaded probes into live rats to demonstrate outgrowth into the hippocampus. This set of experiments will also provide information about whether cells escape from wells *in vivo* at the same rate as *in vitro*.
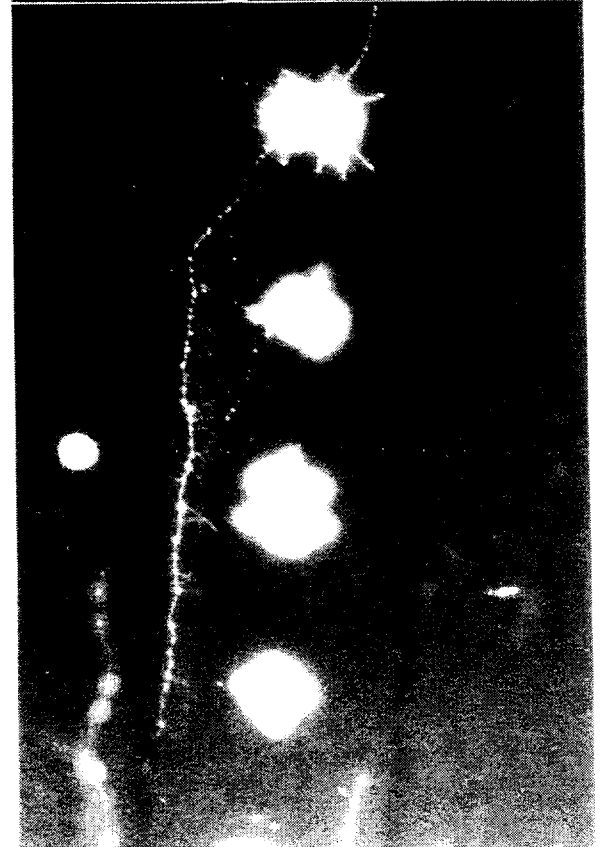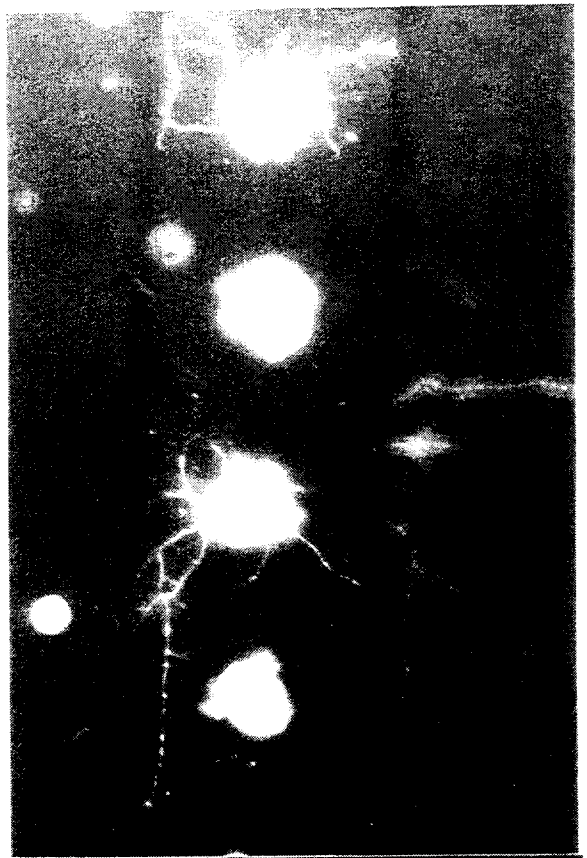
Microfabrication

As noted in the last quarterly report, a run of dummy neurochips was created in an attempt to find a grillwork design that would successfully hold neurons within the wells. Below are some photos of the fabricated wells as taken through a microscope.

Microscope Photos of Fabricated Neuron Wells with Test Grillwork

5μm Grid-Design   5μm Wide Slot-Design
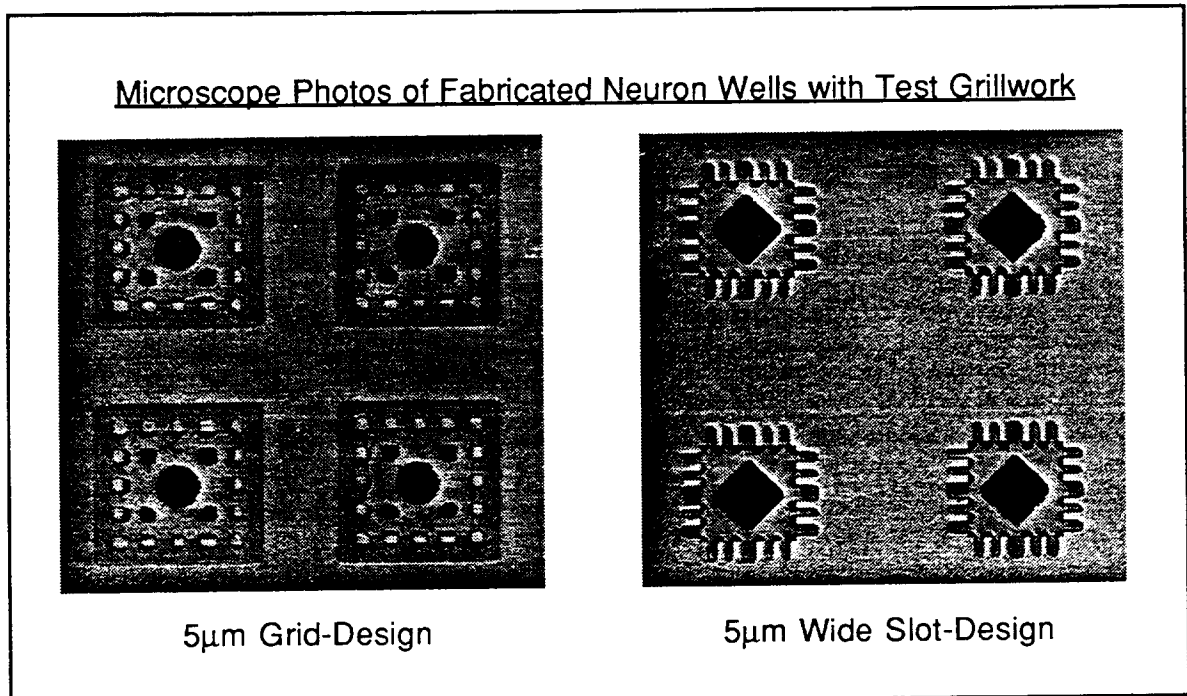
Three weeks of testing has shown that none of the designs adequately restrain neurons within the wells although they are an improvement over the previous design. It was hoped that grillwork with hole sizes of less than 3μm would prove totally successful but that has not been the case. Test with the original grillwork which have markedly larger egress holes for neuron processes, were shown to contain the neuron bodies for less than a week. The new grillwork designs do better as neurons grow for about three weeks before escaping from the wells. Total area of a hole through which a neuron escapes does not appear to be the main factor determining whether it will be capable of constraining a neuron. Rather, the length, i.e. the distance between the grillwork and the well's side (see figure below), is more important. In the grillwork study we conducted, this dimension is created by controlled EDP over-

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etching. Thus, while the widths of the holes were different for different arrays, the lengths for all were approximately the same at ≈ 1μm. This explains why all variations of the designs, each with holes of different areas, hold neurons within their wells for the same length of time.

Unfortunately, three weeks average time is not long enough to permit useful research on the neural networks that are formed. A new grillwork design is needed that will constrain neurons indefinitely. The ZORO (Zero Overhang via Removable Oxide) technique used in the previous grillwork tests can not be modified sufficiently to produce grillwork that will perform much differently than those already studied. As a result, we have proposed a new fabrication process that we hope will be able to keep neurons in the wells while still permitting processes to grow out freely.

The main difference between the ZORO grillwork and the proposed grillwork is the path that the neuron processes take in escaping from the wells. In the ZORO design, the processes crawl up the sides of the well, pass through holes and reach the substrate. To do this, we showed that an overhang of more than 0.5μm of the grillwork around the perimeter of the well is sufficient to greatly inhibit the ability of the processes to successfully escape. As a result, we designed the ZORO process to guarantee that no overhang would be present. We assumed that by making holes small enough, stuffed neurons would be unable to escape while their processes would still be able to grow out. The figure below shows how we had hoped the grillwork would function. Unfortunately it did not prevent escape.
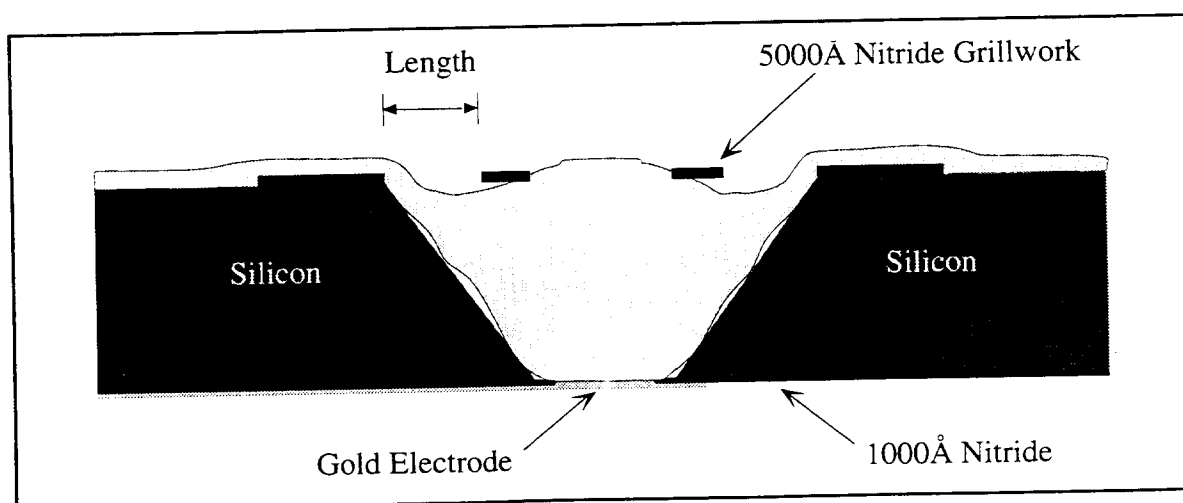
The new technique changes the nitride grillwork into a nitride canopy covering long micro-channels through which processes must grow. The cross section of the channels can be made much smaller than any hole made through the nitride and will hopefully be too small and too long for neuron bodies to crawl through. An additional possible advantage of the channels is that their walls will present a larger frictional barrier between the neuron processes and their bodies. As the processes grow, they pull themselves along and in doing so pull on their cell body. It is this action that pulls the neuron out of its well. The channels may hopefully lessen the pull on the neuron. Instead, the pull that is exerted will be presented to the channel walls only and the neuron body will remain in the well.

Below is shown a cross section of a well along the length of its channels. It shows how the processes still grow up the sides of the well but that it differs from the ZORO design because they must first grow the length of the channel before being free of the well structure. A second important difference is the fact that the canopy must overhang the well in order for the processes to be forced to enter the channels. Remember that the ZORO process was designed specifically to elominate this type of overhang. And so we have come in a way full circle round.
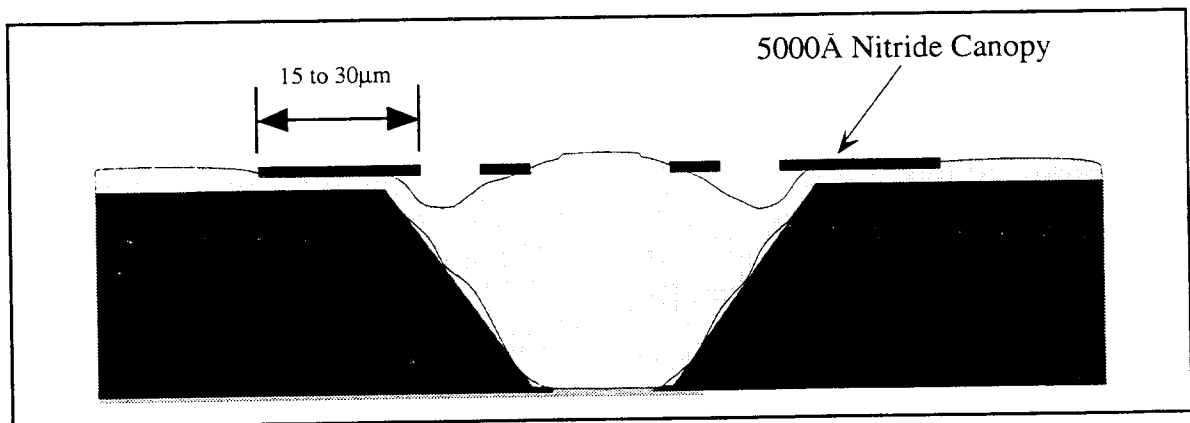

The following figure shows the two basic designs that will be used to test the new fabrication process. The first step is to deposit a layer of PSG (poly silicate glass) on a wafer. This is patterned with the channels mask shown below. Nitride is then deposited and patterned with the canopy mask that is

also shown below. An EDP etch follows to form the neuron wells and finally, the PSG is removed by 49% HF, cleaning out the channels beneath the nitride canopy.

Staggered Nitride Canopy   Staggered Nitride Canopy

Straight Channels   Staggered Channels
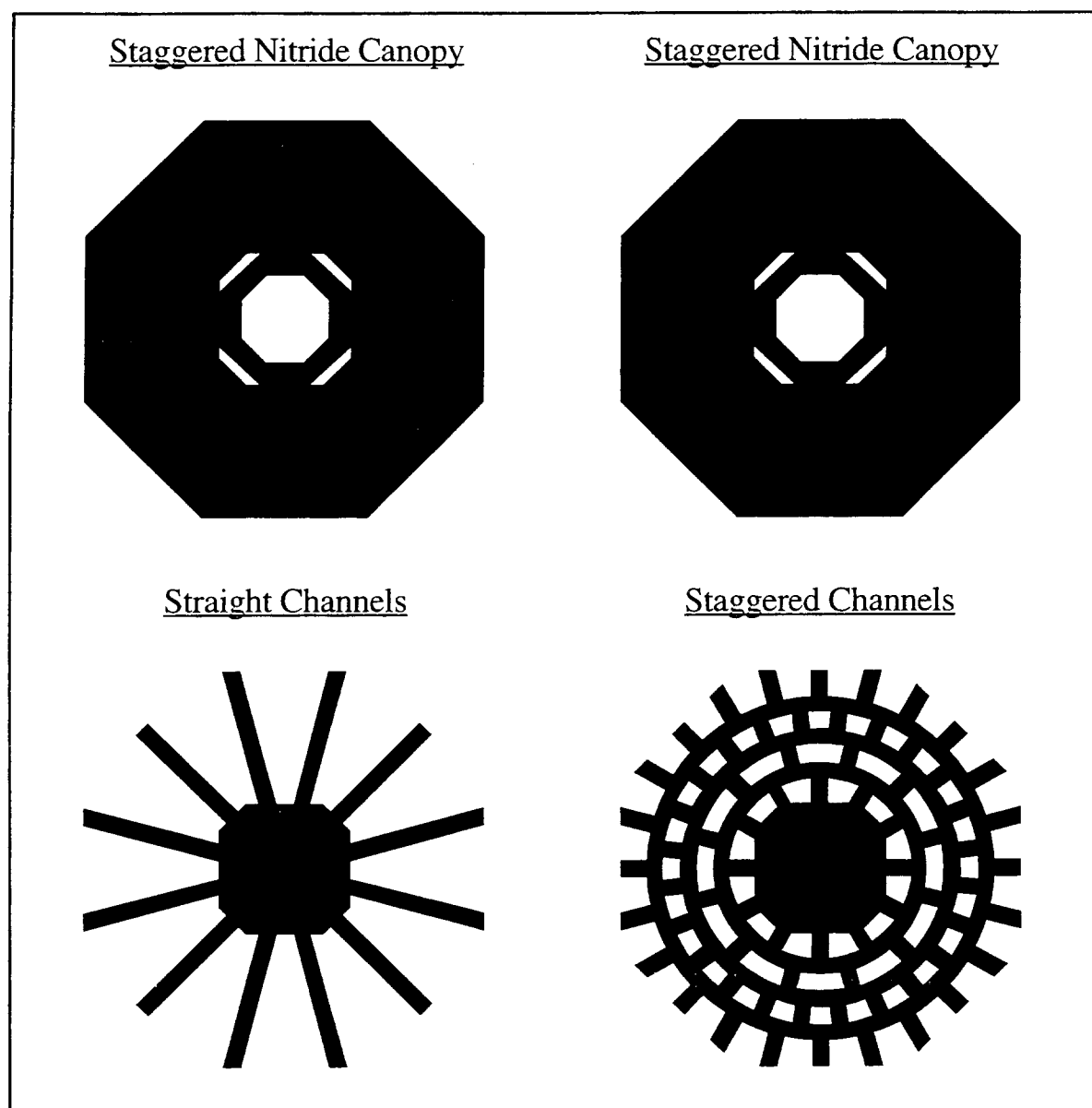
As noted above, nitride is deposited on top of patterned PSG. Effectively this result in the nitride film, the canopy, being "draped" over beams of PSG and down into depressions in the silicon substrate that are a result of the PSG patterning. The profile shown below shows the final cross section of a channel across its width. The minimum width of the channels, as indicated on the figure

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as ≈1.5μm, is limited by the minimum feature size of our photolithography equipment and on the PSG patterning method. The minimum height of the channel can be controlled down to much finer dimensions. Since it is controlled by the thickness of the PSG deposited, it can be as small as 0.2μm.

Channel Cross Section

1.5 to 10μm wide

Nitride Canopy ≈ 0.5μm Thick

Height
0.20 to 3.0 μm

Silicon Substrate
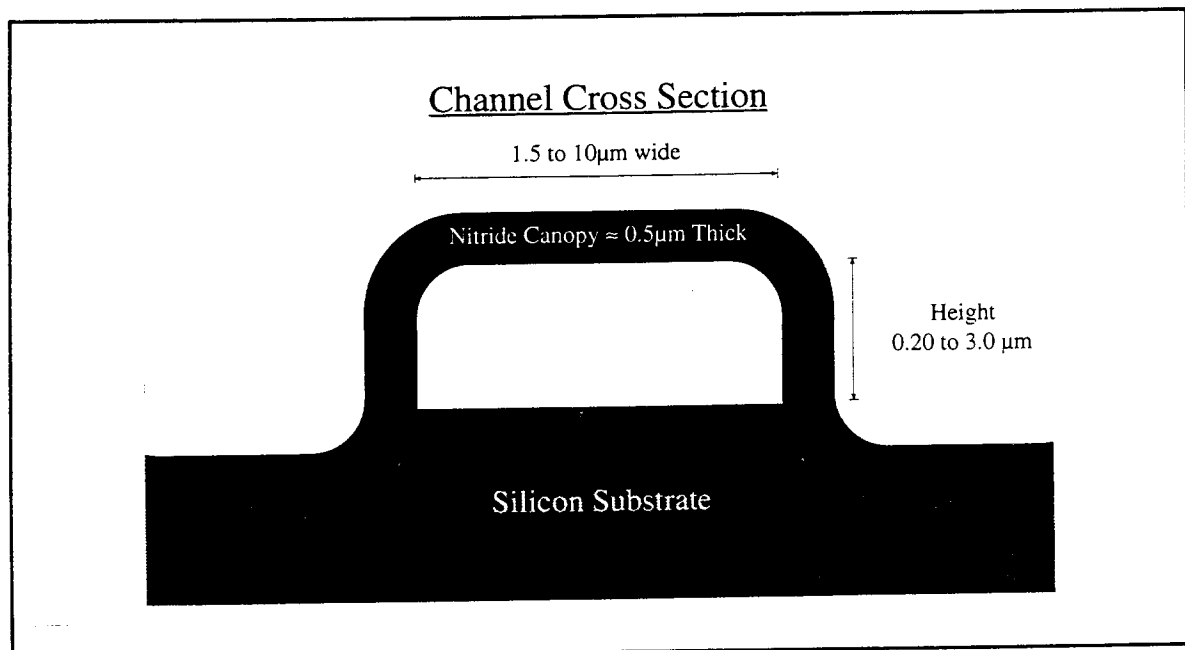
At present, a batch of wafers is being processed using the new fabrication procedure. The mask set incorporates both straight and staggered channels designs, channel widths of 1.5, 2.0, 4.0 and 10μm and channel lengths of 15 and 30μm. The masks will therefore have 16 different arrays of wells. In addition to these, the wafers that will be patterned will have PSG thicknesses of 0.2, 0.3, 0.5, 1.0 and 3.0 μm. The end result will be a daunting total of 80 variations of dummy chips that will need to be tested to see which design will be best suited at keeping the neurons in their wells.

In Vivo Studies

Transplantation of embryonal hippocampal cell suspension stained with DiI into hippocampus of adult rats:

The experiments studying long-term survivability of cells stained with DiI were continued. During this period recipients with grafts were examined eight months after transplantation. Two rats with four grafts were selected for analysis. All grafts were found after eight months. The neurons were still stained with DiI. The dendrites and axons were visible for some of the cells.

Staining Procedure

1. DiI stock solution (40 mg/ml DiI/DMF + 2.5% pluronic F127) and 2 ml serum free Neurobasal/B27 were warmed in 37°C water bath.
2. 1 µl warmed DiI stock solution was pipetted into 2 ml warmed media.
3. Suspension from six-eight hippocampi of 17-day rat embryos was pipetted into stain media.
4. The tube with suspension was placed into incubator for 60 min, and swirled every 5-10 min.
5. The tube with suspension was centrifuged at 200 g for 3 min.
6. Supernatant was pipetted off, 1 ml Neurobasal/B27 medium was added and a cell pellet was resuspended.
7. The steps 5 and 6 were repeated and cells were left at room temperature after additional centrifugation till transplantation.

Transplantation Procedure

Sprague-Dawley rats were anesthetized with a mixture 4 mg/kg of ketamine (25 mg/kg), xylazine (1.3 mg/kg) and acetopromazine (0.25 mg/kg) and fixed in a stereotaxic frame. 0.3-0.5 mm^3 of suspension taken from the pellet were transplanted into the left and right hippocampus by microsyringe, after opening the skull.
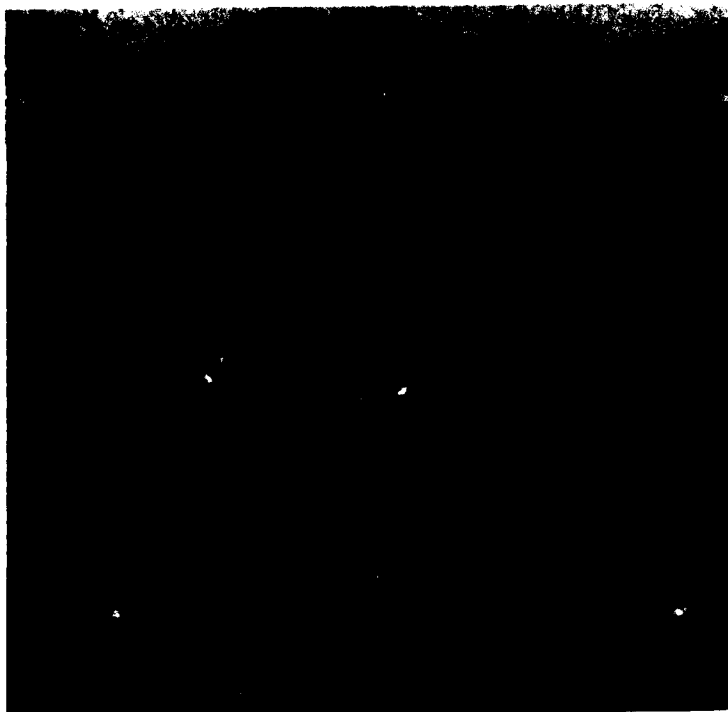
Observations

For later examination of the transplants, rats were deeply anesthetized and perfused with 200 ml of saline followed by 10% of buffered formalin. Then brains were cut on a vibratome in 60 μm sections, and examined under a fluorescent microscope.

Two rats received stained suspension cell transplants. Nine months later transplants were found in all tested rats. Their volumes did not exceed 1 mm^3. As noticed in our previous reports, we have revealed stained cells one month, and six months after the transplantation. At eight months after transplantation the stained cells looked healthy and the dye was concentrated in the cell membrane, as shown in the figure below. **A** is an unstained section, at low magnification. **B** is at a higher magnification. **C** is a fluorescent view of B, with many labeled cells.

The next figure shows a second example. **A** shows Nissal staining of the transplant. **B** is a fluorescent view of the same section. **C** and **D** are two DiI labeled neurons with processes. The density of DiI fluorescence varied in different cells. In some cells it was weak, and in others very bright; and in a few cells both axons and dendrites could be recognized, as shown in the second figure below. A small amount of glia accumulated DiI. DiI was also revealed in nearby vessels.
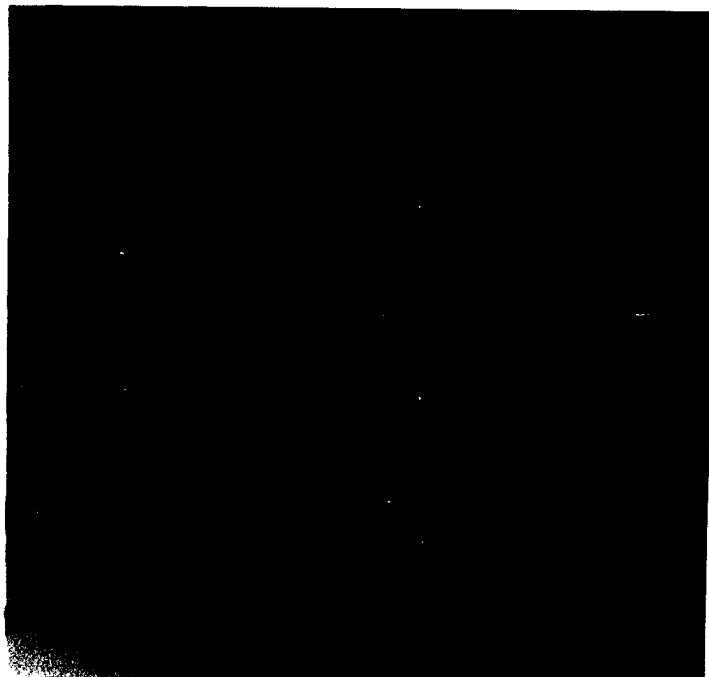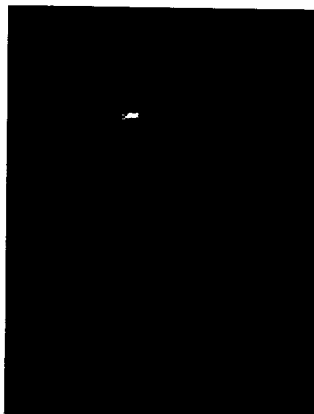
A

B

C

A

B

C

D